

REMARKS

The presently claimed invention concerns a method for biasing the amplification of the DNA molecules in a sample such that a nucleic acid molecule having a specific nucleotide at a selected position (e.g., a polymorphic site) is preferentially amplified relative to an otherwise identical nucleic acid molecule not having the specific nucleotide at the selected position. The method is useful for preferentially amplifying at least a portion of one allele of a gene relative to another, different allele of the gene in a sample containing both alleles of the gene.

It is often desirable to obtain a relatively pure sample of one allele of a gene. For example, where an individual harbors two different alleles of a gene of interest, haplotyping the gene is practical only if one can obtain a relatively pure sample of one of the two different alleles of the gene of interest. The methods of the invention provide a means for obtaining a relatively pure sample of one of two different alleles of a gene in a DNA sample containing both alleles through the use of a so-called biased amplification reaction that greatly favors amplification of one of the two alleles present in the sample. For example, the method of the invention can be used to preferentially amplify the maternal allele of a gene of interest relative to the paternal allele where the two alleles differ in sequence at a polymorphic site. Thus, the methods of the invention can be used to obtain a relatively pure sample of either allele for use in haplotyping.

The method of the invention relies, in part, on the fact that the presence of a stable stem-loop structure in a nucleic acid molecule can inhibit amplification (e.g., PCR amplification) of the molecule if the stem-loop is relatively stable.

Rejections Under 35 U.S.C. §112, second paragraph

The Examiner rejected claim 1 under 35 U.S.C. §112, second paragraph as allegedly indefinite. According to the Examiner, claim 1 is unclear for failing to describe how amplification is achieved and is confusing because the claimed method "is not sufficiently set forth." Claim 1 has been cancelled, obviating this rejection.

The newly added claims are believed to be clear and complete. Accordingly, Applicant respectfully requests that the rejections under 35 U.S.C. §112, second paragraph be withdrawn.

Rejections Under 35 U.S.C. §103

The Examiner rejected claims 1-9 under 35 U.S.C. §103 as obvious in view of Bolla et al. (*Clin. Chem.* 41:1599, 1995) and Caetano-Anolles et al. (*Biotechniques* 20:1044, 1996). According to the Examiner it would have been obvious to “combine the method of detecting genotypes of apoE gene using PCR-RFLP as taught by Bolla et al. with the method of Caetano-Anolles et al. which is applicable to achieve rapid identification of alleles.”

Applicant respectfully disagrees with the Examiner's assessment of the prior art. Neither cited reference teaches a method for biasing an amplification reaction such that one nucleic acid molecule having a first nucleotide at a selected position is preferentially amplified relative to a second nucleic acid molecule having a different nucleotide at the selected position. Moreover, modification of the method of Bolla et al. to use the hairpin primers of Caetano-Anolles et al. would not result in a method for performing biased amplification.

The cited references no matter how combined do not provide the elements of the present claims. Moreover, it would be illogical to combine the methods of Bolla et al. and of Caetano-Anolles et al.

Before discussing the deficiencies of the cited prior art, it is useful to review the manner in which the presently claimed methods permit one to preferentially amplify a nucleic acid molecule having a specific nucleotide at a selected position. One embodiment of the presently claimed methods is described below. The description includes schematic drawings that are based on Figures 14-17 of the specification, but they have been simplified somewhat for the sake of greater clarity.

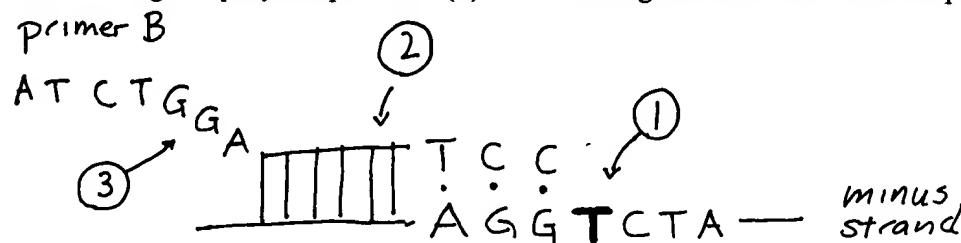
Consider a gene of interest that has a polymorphic site at a known position. The nucleotide at this position is “T” in the case of one allele (“the ‘T’ allele”) and “C” in the case of the other allele (“the ‘C’ allele”). A portion of the minus strand of the “T” allele is depicted below. The polymorphic “T” (1) is shown in **bold**. In the drawings only a few nucleotides in the area of the polymorphic site are explicitly depicted and some base-pairs are depicted by a vertical line.

—AGG**T**CTA— *minus strand*

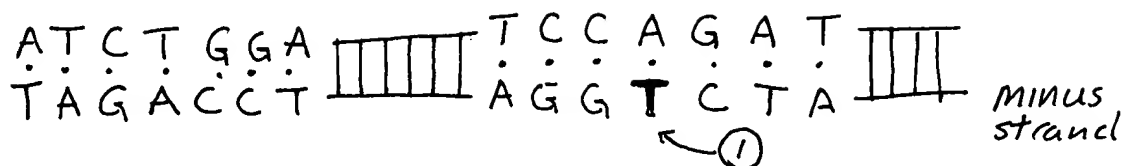
A subject is known or thought to be heterozygous at the this polymorphic site, and it is considered desirable to haplotype the patient by obtaining a relatively pure sample of the "C" allele. This can be achieved by using the presently claimed methods. In this instance specially designed primers that permit amplification of the "C" allele while inhibiting amplification of the "T" allele are used as follows.

A sample of DNA taken from the patient is subjected to PCR amplification using two primers. The first primer (Primer A) binds to the plus strand (not shown) and is a conventional primer. The second primer (Primer B) binds to the minus strand and is designed to incorporate sequences into the amplification product that cause the formation of a perfect (relatively stable) stem-loop structure upon amplification of one allele, in this case the "T" allele, and forms an imperfect (relatively unstable) stem-loop structure upon amplification of the other allele, in this case the "C" allele.

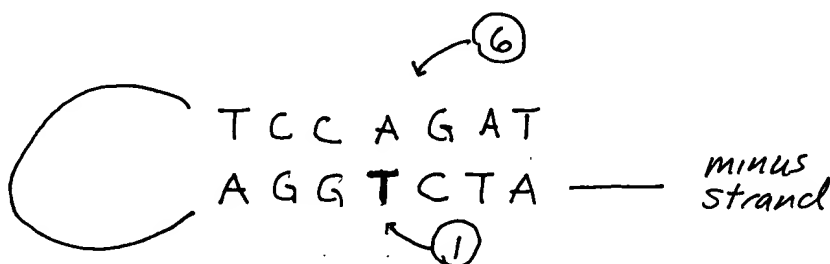
The sketch below depicts the initial binding of Primer B to the minus strand of the T allele. A 3' portion (2) of Primer B is complementary to the minus strand. A 5' portion (3) of this primer is not complementary to the minus strand, although after two rounds of amplification, this portion will be incorporated into the amplification product. The 5' portion that is not complementary to the minus strand (3) includes a sequence that will, during amplification, cause the incorporation into the minus strand of a sequence that can base-pair with the region surrounding the polymorphic site (1) thus forming the stem of a stem-loop.



After two rounds of amplification, the amplification product has the structure shown below. The 5' portion of the primer (3) has become incorporated into the amplification product, and it can be seen that the incorporation of the primer sequence has led to the incorporation into the minus strand a portion (4) that will form the stem of the stem-loop. This portion is perfectly complementary to the region surrounding the polymorphic site (1). Thus, it includes an "A" (5) that can base-pair to the "T" at the polymorphic site (1).



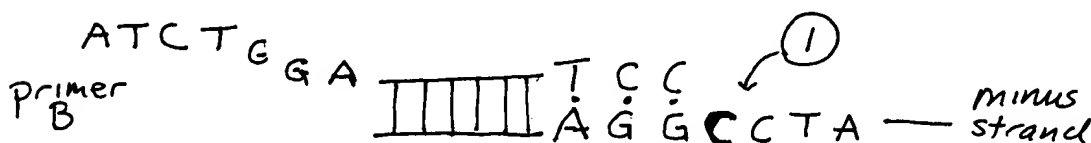
During the next round of denaturation and annealing, the minus strand of the amplification product forms a stem-loop structure, depicted below, having a perfectly matched stem (6). This stem includes the polymorphic site T (1). The stem-loop structure is stable enough to inhibit further amplification of the T allele relative to the C allele.



The situation is different for the C allele. This allele, depicted below, differs from the T allele in that a "C" is present at the polymorphic site (1).

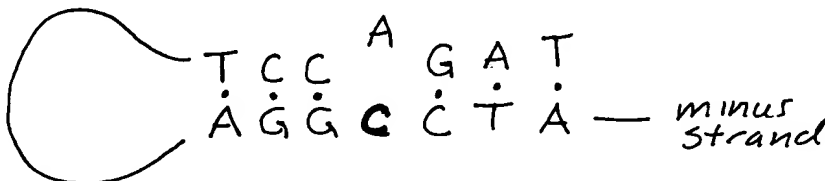


As depicted below, Primer B binds to the "C" allele just as it binds to the "T" allele.



After two rounds of amplification, the 5' region of the primer is incorporated into the amplification product. Of course, in this case, the very 5' end of the minus strand is not perfectly complementary to the region surrounding the polymorphic site. This is because the primer was designed to be perfectly complementary to the region of the polymorphic site only when a "T" is present at the polymorphic site, not when a "C" is present.

Thus, upon the next round of denaturation and annealing, the stem of the stem-loop that forms is imperfect, as shown below. It contains a mis-match at the polymorphic site. This stem loop is not stable enough to significantly inhibit further amplification.



As a result, "C" allele nucleic acid molecules undergo additional amplification while amplification of "T" allele nucleic acid molecules is inhibited. As a result of the processes described above, amplification with Primer A and Primer B produces amplification product that is greatly enriched for copies of the "C" allele. This amplification product can be used to haplotype the "C" allele without excessive interference from the "T" allele.

With this understanding of the claimed methods, the deficiencies of the prior art can be clearly understood.

Bolla et al.

Bolla et al. describe a restriction digestion method for determining the genotype of an individual's ApoE gene. In the case of the ApoE gene studied by Bolla et al., each of two polymorphic sites, one at nucleotide 112 and the other at nucleotide 158, alters the cleavage pattern observed upon HhaI digestion (see Figure 1 of Bolla et al.). Thus, as Bolla et al. explain, PCR amplification followed by HhaI digestion and fragment sizing by gel electrophoresis can be used to distinguish the six most common genotypes of the ApoE gene. As Bolla et al. point out, the haplotyping of an ApoE gene using restriction digestion is possible in the case of positions 112 and 158 because both of the polymorphic sites alter the site of restriction digestion and because the size of the fragments produced by restriction digestion are such that the various genotype can be deduced. The method described by Bolla et al. does not depend upon preferential amplification of one allele. The two primers used in the amplification reaction more or less equally amplify all alleles present in the amplification reaction. It is the subsequent

restriction digestion that is used to distinguish certain genotypes. This is very different from the presently claimed method.

The presently claimed methods permit amplification of an allele having one particular nucleotide at a polymorphic site while substantially inhibiting amplification of a second, different allele. One advantage of this biased amplification is that one can obtain amplification product that contains very, very many copies of one allele and relatively few copies of the other allele. Bolla et al. do not carry out a biased amplification reaction. The primers used by Bolla et al. amplify all alleles essentially equally. Indeed, this is a requirement of the method of Bolla et al. This is because Bolla et al. distinguish genotypes based on the size pattern of fragments produced by restriction digestion of the amplification products. If one allele were amplified poorly or not at all, there would be too little of the diagnostic restriction fragments for proper analysis.

Thus, there are at least two significant differences between the method of Bolla et al. and the presently claimed method. First, Bolla et al. do not perform a biased amplification reaction that results in the production of amplification products that contain far more of one allele than another allele. Second, the primers used by Bolla et al. are both perfectly matched to the ApoE gene along their entire length. This is unlike the present method in which one primer has 5' region that does not hybridize to the initial target template.

Caetano-Anolles et al.

Caetano-Anolles et al. describe a variation on a standard DNA fingerprinting technique that relies on PCR amplification using random primers to create a "fingerprint" that can be used, for example, to distinguish DNA samples derived from two closely related plants.

In the variation described by Caetano-Anolles et al. are similar to standard fingerprint primers in that they have a portion that is as few as three nucleotides long and has a random (arbitrary) sequence. In addition, the primers used by Caetano-Anolles et al. have a 5' portion that forms a small and stable hairpin loop. For example, one primer has the sequence GCGAAGCNNN (where N is any nucleotide). The NNN portion binds randomly throughout the DNA molecule being amplified, and the GCGAAGC portion is forms a hairpin loop. The presence of the hairpin loop, which is incorporated into all extension products, causes the

products to have very high electrophoretic mobility. As Caetano-Anolles et al. explain, the presence of a hairpin loop in the primer results in the production of far more (25%-80% more) extension products in the 50 – 700 basepair size range than do primers lacking a hairpin loop. For this reason, Caetano-Anolles et al. suggest that the mini hairpin primers are useful for generating fingerprint patterns of plant DNA that can distinguish taxa at the interspecies and intraspecies level (see text above Figure 3).

The differences between the method of Caetano-Anolles et al. and the presently claimed methods are clear. First, as can be seen from the forgoing discussion, Caetano-Anolles et al. do not perform a biased amplification reaction that results in the production of amplification products that contain far more of one allele than another allele. In fact, since the method of Caetano-Anolles et al. uses random primers, all alleles of every gene are amplified more or less equally. Second, the primers described by Caetano-Anolles et al. include a stable hairpin at the 5' end. The primers used in the present method do not include such a hairpin loop.

The combination of Bolla et al. and Caetano-Anolles et al.

As the forgoing discussion makes clear, neither of the cited references provide a method for achieving biased amplification such that one nucleic acid molecule having a first nucleotide at a selected position is preferentially amplified relative to a second nucleic acid molecule having a different nucleotide at the selected position. Moreover, adding the mini-hairpin loop found in the primers of Caetano-Anolles et al. to the primers of Bolla et al. would not lead to biased amplification since there is nothing about either the primers of Caetano-Anolles et al. or the primers of Bolla et al. that distinguish between two nucleic acid molecules having a different nucleotide at a polymorphic site.

In any event, it would be illogical to use the hairpin primers of Caetano-Anolles et al. in the method Bolla et al. because the hairpins in the primers of Caetano-Anolles et al. appear to be “involved in selecting annealing sites during the primer-template screening phase of the amplification reaction” (Caetano-Anolles et al., page 1054, right column). Bolla et al. uses selective primers to amplify a portion of the ApoE gene. It would be highly undesirable to modify these selective primers by adding hairpins, such as described by Caetano-Anolles et al., that might alter the annealing site of the primers.

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In sum, even if there were some reason to combine the teachings of the references (there is not), adding the mini-hairpin loop of Caetano-Anolles et al. to the primers of Bolla et al. would not result in a primer having the ability produce amplification products "that form a stable hairpin loop the stem of which is perfectly matched and includes the polymorphic site only when the second nucleotide is present at the polymorphic site" as required by the present claims.

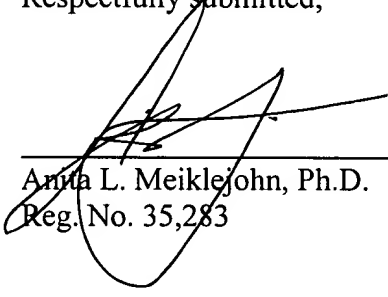
In view of the forgoing, Applicant respectfully requests that the rejections under 35 U.S.C. §103 be withdrawn.

Conclusion

Applicant asks that all claims be allowed. A Petition for Extension of Time with the appropriate fee is enclosed. Please apply any other charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

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Version with markings to show changes made

In the Specification:

Paragraph beginning at page 27, line 19, has been amended as follows:

--**Figure 1.** Depiction of a primer designed to incorporate restriction enzyme recognition sites for the specific restriction enzymes Fok I and Fsp I. The primer (primer R sequence) has altered bases from the desired amplified region of the target DNA. The polymorphic nucleotide is included in the target DNA region and is [as indicated by the arrow]underlined. After PCR amplification, the incorporated altered base pairs of the primer thereby incorporate FokI and FspI restriction sites in the amplicon. The amplicon can subsequently be digested in the presence of the FokI and FspI restriction enzymes under optimal conditions for digestion by both enzymes. The resultant fragments after enzyme digestion, an 8-mer and a 12-mer, are as depicted. In this figure, the polymorphism (A, [in *italic*]underlined) is contained within the 12-mer fragment.--

Paragraph beginning at page 28, line 8, has been amended as follows:

--**Figure 4.** In this figure, an alternative method of primer design in the present invention involves the use of a primer with an internal loop. The primer is designed (primer R1) such that one of the bases corresponding to the native sequence is removed and replaced with a loop. In this case the G/C indicated by the arrow below the target sequence is replaced with the recognition sequence for Fok I and Fsp I. Upon hybridization to the DNA template, the primer will form a loop structure. This loop will be incorporated into the amplicon during the amplification process, thereby introducing the Fok I and Fsp I restriction sites (indicated by the box). The resultant amplicon is incubated with Fok I and Fsp I under optimal digestion conditions producing an 8-mer and a 12-mer fragment. As in Figure 1, the 12-mer contains the polymorphic base (A [in *italic*]underlined) and can be analyzed by mass spectrometry to identify the base at the polymorphic site.--

Paragraph beginning at page 28, line 20, has been amended as follows:

--**Figure 5.** Alternative restriction enzyme recognition site incorporation into amplified regions of target DNA is shown. As is depicted in figures 1-4 for the enzyme pair FspI/FokI; in this figure, PvuII/FokI restriction enzymatic sites can be incorporated in the same manner as

previously described for Figures 1-4. A primer is designed such that a BsgI/PvuII sites form a hair-pin loop when the primer is hybridized to the target DNA sequence. After amplification by PCR, the resultant amplicon will have the PvuII/FokI sites incorporated in the resultant amplicon (as indicated by the boxed sequence). After digestion under conditions optimal for PvuII and BsgI, the resultant fragments, an 14 mer and a 16 mer, are sufficient for mass spectrometric analysis and the polymorphic site is contained in the 16mer (A, [in italic]underlined).--

Paragraph beginning at page 28, line 30, has been amended as follows:

--**Figure 6.** Shown in this figure is an alternative restriction enzyme pair for the preparation of fragments containing the polymorphic site for mass spectrometric analysis. PvuII/FokI restriction enzyme recognition sites form a hair-pin loop when hybridized to the target DNA sequence. After amplification by PCR, the resultant amplicon will have the PvuII/FokI sites incorporated in the resultant amplicon (as indicated by the boxed sequence). After digestion under conditions optimal for PvuII and FokI restriction, the resultant fragments, an 16 mer and a 20 mer, are sufficient for mass spectrometric analysis and the polymorphic site is contained in the 20mer (A, [in italic]underlined).--

Paragraph beginning at page 29, line 26, has been amended as follows:

--**Figure 9.** Shown in this figure is an example of the utility in the present invention of including a restriction enzyme recognition site for which the restriction enzyme creates a nick in the DNA amplicon instead of causing a double strand break. As shown in this figure, a primer R is designed to incorporate a N.BstNB I recognition site (GAGTCNNNN[^]NN) in addition to a FokI restriction site. As in previous figures, the primer forms a hair-pin loop structure when hybridized to the target DNA region, however, the PCR amplicon has the incorporated restriction site sequences. Digestion with FokI and N.BstNB I results in a 10 mer fragment that contains the polymorphic base (T, [in italic] underlined). Such a fragment is sufficient for analysis using a mass spectrometer.--